METHODS FOR DETECTING CANCER CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to a highly sensitive assay for distinguishing between cancer and non-cancer epithelial cells in the blood that provides an improved diagnostic technique for detecting cancer and determining the organ-origin of the cancer

Background Art

Early detection is the hallmark of successful cancer treatment. However, many tumors remain clinically occult until they are far advanced. The reasons for this are varied. For example, the remote anatomical location of the pancreas makes it unlikely that pancreatic carcinomas will be detected before they have invaded neighboring structures. Although the breast is anatomically accessible, breast cancers metastasize very early in their course; consequently, 12-37% of small (<1 cm) breast cancers detected by mammography already have metastasized at diagnosis (*Wilhelm et al.* 1991. "Nonpalpable invasive breast cancer" Ann. Surg 213: 600-605 and *Chadha et al.* 1994. "Predictors of axillary lymph node metastases in patients with T1 breast cancer." Cancer 73: 350-353). Even in prostrate cancer, in which a prostate-specific antigen can be quantified in the serum, there is a substantial percentage of patients with elevated PSA in whom diagnosis remains uncertain. Thus, the urologist must decide whether a biopsy is necessary, or, if a biopsy has been performed and is negative, if and when it should be repeated. These examples indicate that there is a need for improved diagnostic techniques for cancer.

There is some preliminary evidence that primary cancers begin shedding neoplastic cells into the circulation at an early stage (*Butler and Gullino* 1975. Cancer Res. 35: 512-516; *Glaves and Mayhew* 1984. Cancer Drug Delivery 1: 293-302; *Liotta et al.* 1974. Cancer Res. 34: 997-1004 and *Fidler* 1973. Eur. J. Cancer 9:223-227). However, the natural history of these cells, their ability to establish metastases, and their bearing on future relapses are unclear. If tumor cell shedding is, in fact, an early

event in tumorigenesis, it may be possible to detect cancer cells in the bloodstream before the primary tumor is large enough to be detected by standard screening examinations.

This invention provides a highly sensitive assay for detecting carcinomas much earlier than conventional assays. This assay can be used to prove the neoplastic nature of cells and predict when shed tumor cells have or will become metastatic. Furthermore, the assay provides an early detector of treatment success or failure and thereby allows a treatment regimen to be customized for an individual patient with advanced primary cancer.

Therefore, this invention provides a highly sensitive assay for detecting, enumerating and characterizing epithelial cells in the blood. The assay has been used to detect one such cell in 2 X 10^8 leukocytes. The method allows characterization of the cells for their malignant nature, genetic alterations, organ of origin and aggressiveness. This assay will detect carcinomas at a very early stage and aid prognostication. Circulating cancer cells can be detected in high risk individuals before detection of the primary tumor and the assay can be used to monitor therapy. Therefore, the present invention fulfills the need for an improved diagnostic technique for detecting, treating, and prognosticating cancer.

SUMMARY OF THE INVENTION

This invention provides a method of screening for the presence of a cancer cell, comprising obtaining a cell from a subject, contacting the cell with a probe capable of hybridizing to a nucleic acid of the cell, and detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell, thereby screening for the presence of a cancer cell.

The invention also provides a method of screening for the presence of a cancer cell, comprising obtaining a biological sample from a subject, wherein the biological sample comprises a mixed cell population suspected of containing a population of epithelial cells which include a cancer cell; mixing the biological sample with magnetic particles coupled to a ligand which is capable of reacting specifically with epithelial cells to the substantial exclusion of non-epithelial cells; enriching the biological sample for epithelial cells by subjecting the biological sample mixed with magnetic beads to a magnetic field to produce a cell suspension that is enriched epithelial cells; contacting the enriched cell suspension with a probe capable of hybridizing to a nucleic acid of the cell; and detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell, thereby screening for the presence of a cancer cell.

Also provided by the present invention is a method of determining the status of a cancer comprising obtaining a biological sample containing a cell from a patient diagnosed with cancer, contacting the cell in the sample with a probe capable of hybridizing to nucleic acid of the cell, detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell, determining the amount of cancer cells in the sample; and correlating the amount of cancer cells in the sample with a stage of cancer, thereby determining the status of the cancer.

The invention also provides a method of determining the status of a cancer comprising obtaining a biological sample containing a cell from a patient diagnosed with cancer; contacting the cell in the sample with a probe under conditions capable of forming a complex with an antigen of the cell; detecting the complex, whereby detection of the complex can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample; and correlating the amount of

cancer cells in the sample with a stage of cancer, thereby determining the status of the cancer.

Further provided is a method of determining the progression of a cancer comprising obtaining a biological sample containing a cell at a first time point from a patient diagnosed with cancer and obtaining a biological sample containing a cell from the patient at a second time point; contacting the cell in the first sample and the cell in the second sample with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in both the first sample and the second sample; and comparing the amount of cancer cells in both the first sample and the second sample, whereby the relative amount of cancer cells in the first sample as compared with the second sample may be correlated with the progression of cancer, thereby determining the progression of the cancer.

In another embodiment the invention provides a method of determining the progression of a cancer comprising obtaining a biological sample containing a cell at a first time point from a patient diagnosed with cancer and obtaining a biological sample containing a cell from the patient at a second time point; contacting the cell in the first sample and the cell in the second sample with a probe under conditions which allow the probe to form a complex with an antigen of the cell; detecting the complex in both the first sample and the second sample, whereby detection of the complex can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the first sample and the second sample; and comparing the amount of cancer cells in both the first sample and the second sample, whereby the relative amount of cancer cells in the first sample as compared with the second sample may be correlated with the progression of cancer, thereby determining the progression of the cancer.

The invention also provides a method of determining the effectiveness of an anti-cancer treatment comprising obtaining a biological sample containing a cell from a patient that has been administered an anti-cancer treatment; contacting the cell in the sample with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with the effectiveness of the anti-cancer treatment, thereby determining the determining the effectiveness of an anti-cancer treatment.

Also provided is a method of determining the effectiveness of an anti-cancer treatment comprising obtaining a biological sample containing a cell from a patient that has been administered an anti-cancer treatment; contacting the cell in the sample with a probe under conditions capable of forming a complex with an antigen of the cell; detecting the complex, whereby detecting the complex can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with the effectiveness of the anti-cancer treatment, thereby determining the determining the effectiveness of an anti-cancer treatment.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows selection of epithelial cells in a blood sample. The large, red stained cells indicate the presence of epithelial cells in peripheral blood. The cells were stained with an antibody against keratin (arrows). The smaller, unstained cells (arrowheads) represent lymphocytes.

Figure 2 shown *in situ* hybridization of nucleic acid of a cell using a fluorescently labeled DNA probe for the centromere of chromosome 8. The two

signals in the cytokeratin negative cell reflect the diploid copy number expected in normal lymphocytes. Four signals in the cytokeratin positive epithelial cells clearly indicate the presence of chromosomal aneuploidy, which is specific for cancer. In this particular case, the aberration matches with the known cytogenetic profile of this breast carcinoma.

Figure 3 shows a sample containing two cells from a patient with a prostate cancer. The cells were hybridized to centromer probes for chromosome 7 (red) and for chromosome 8 (green). The larger cell is a tumor cell which exhibits greater than two fluorescent *in situ* hybridization (FISH) signals whereas the normal cells show only two FISH signals.

Figure 4 shows a sample containing two cells from a woman with breast cancer. The cells were hybridized to centromer probes for chromosome 7 (red) and for chromosome 8 (green). The larger cell is a tumor cell which exhibits greater than two fluorescent *in situ* hybridization (FISH) signals whereas the normal cells show only two FISH signals.

DETAILED DESCRIPTION OF THE INVENTION

The present invention may be understood more readily by reference to the following detailed description of the preferred embodiments of the invention and the Examples included therein.

Before the present methods are disclosed and described, it is to be understood that this invention is not limited to specific probes, specific methods, or specific nucleic acids, etc. as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an", and "the" include plural referents unless the context clearly dictates otherwise. For example, "a sample containing a cell" can mean one or more samples, each containing one or more cells.

This invention provides a method of screening for the presence of a cancer cell, comprising obtaining a cell from a subject, contacting the cell with a probe capable of hybridizing to a nucleic acid of the cell, and detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell, thereby screening for the presence of a cancer cell.

Each of the methods of the invention described herein may be modified so that the genetic probing step is preceded by one or more steps designed to enrich the sample for a certain cell type. For example, circulating cells may be enriched for epithelial cells by cytokeratin screening. This can be accomplished, for example, by flow cytometry or immunohistochemistry. Since cytokeratin is an epithelial cell specific marker, cytokeratin Mabs can be utilized to enrich a sample for epithelial cells. An example of a screening procedure would be tumor cell enrichment by depletion of CD45-expressing mononuclear cells (MNC) followed by immunocytochemical detection (ICC) for detection of the cytokeratin (CK)-positive (+) epithelial cells. In addition to cytokeratin, other cell specific markers can be utilized to enrich a sample for use in the methods of this invention. The sample can be enriched for any cell associated with tumorigenesis. For example, samples can be enriched by tumor-specific antigen screening, or tissue-specific antigen screening. Examples of tumor-specific antigens include prostate specific antigen (PSA), CA125, Cyfra 21-1 and TPS.

The biological sample may also be enriched for a certain target cell type, such as, for example, epithelial cells, by using the methods described in International Patent Publication W0 99/41613, the contents of which are incorporated by reference herein in

their entirety. In this method, a biological sample comprising a mixed cell population suspected of containing a certain cell type, such as epithelial cells, is obtained from a subject, and the biological sample is then mixed with magnetic particles coupled to a ligand which is capable of reacting specifically with the target cells to the substantial exclusion of non-target cells to form an immunomagnetic sample. Preferably, the biological sample is a fluid; most preferably, the biological sample is blood. A cell suspension enriched for the target cells is then obtained by subjecting the immunomagnetic sample to a magnetic field to produce a cell suspension that is enriched target cells.

Preferably, as is described in International Patent Publication W0 99/41613, the magnetic particles are submicroscopic ferrous particles, as conventional magnetic beads cause non-specific aggregation of the cells and a high background of staining.

In a preferred embodiment, the cell sample that has been enriched for a specific cell type by the immunomagnetic method described above is further subjected to immunocytochemistry prior to being probed with cancer-cell specific nucleic acid probes. Thus, the sample may be treated with a labeled antibody, and then FISH performed on these same samples. Typically, samples that have been immunocyto-chemically stained for bright field microscopy would not be expected to be useful for subsequent FISH analysis (which of course uses fluorescence microscopy) due to interference between the different types of dyes used for these two microscopy techniques. However, using the methods of the invention, it is possible to simultaneously visualize the epithelial origin of the cells and the chromosome count. This provides an internal control for the hybridization and increases the diagnostics accuracy. For example, the cells may be probed with a labeled antibody specific for epithelial cells, such as an antibody specific for cytokeratin, so as to further ensure that the cells being subjected to nucleic acid probing are indeed epithelial cells. The

antibody may be labeled by any of the labeling means described elsewhere in this application.

Thus, the invention also provides a method of screening for the presence of a cancer cell, comprising obtaining a biological sample from a subject, wherein the biological sample comprises a mixed cell population suspected of containing a population of epithelial cells which include a cancer cell; mixing the biological sample with magnetic particles coupled to a ligand which is capable of reacting specifically with epithelial cells to the substantial exclusion of non-epithelial cells; enriching the biological sample for epithelial cells by subjecting the biological sample mixed with magnetic beads to a magnetic field to produce a cell suspension that is enriched epithelial cells; contacting the enriched cell suspension with a probe capable of hybridizing to a nucleic acid of the cell; and detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell, thereby screening for the presence of a cancer cell.

The invention further provides a method of determining the status of a cancer comprising obtaining a biological sample containing a cell from a patient diagnosed with cancer; contacting the cell in the sample with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with a stage of cancer, thereby determining the status of the cancer.

The invention further provides a method of determining the status of a cancer comprising obtaining a sample from a patient diagnosed with cancer, obtaining a biological sample from a subject, wherein the biological sample comprises a mixed cell population suspected of containing a population of epithelial cells which include a cancer cell; mixing the biological sample with magnetic particles coupled to a ligand

which is capable of reacting specifically with epithelial cells to the substantial exclusion of non-epithelial cells; enriching the biological sample for epithelial cells by subjecting the mixture of the biological sample with magnetic particles coupled to a ligand to a magnetic field to produce a cell suspension that is enriched epithelial cells; contacting the enriched cells with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with a stage of cancer, thereby determining the status of the cancer.

The invention also provides a method of determining the status of a cancer comprising obtaining a biological sample containing a cell from a patient diagnosed with cancer; contacting the cell in the sample with a probe under conditions capable of forming a complex with an antigen of the cell; detecting the complex, whereby detection of the complex can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample; and correlating the amount of cancer cells in the sample with a stage of cancer, thereby determining the status of the cancer.

One skilled in the art will readily be able to utilize the methods of this invention to obtain a sample from a patient and determine the amount of cancer cells in the patient As more patients are assayed for a particular type of cancer, more information will be obtained about the number of cancer cells (level of tumor burden) associated with a particular stage of cancer. Ranges of the numbers of cancer cells can be assigned to particular stages of cancer to assist in determining the clinical status of the patient as well as in prescribing a particular treatment method for the patient.

The subjects for prognostic determination include patients who, for example, have a diagnosed breast carcinoma (with or without distant metastasis), patients who

have a diagnosed breast carcinoma and who are scheduled for bone marrow transplantation, individuals from families with high-risk to develop breast carcinoma, individuals from families with BRCA gene mutations, and patients with inflammatory diseases or benign tumors of the breast. The subjects for prognostic determination can also include patients who have been diagnosed or who are at high risk to develop cervical cancer, breast cancer, prostate cancer, lung cancer, pancreatic cancer, brain cancer, ovarian cancer, liver cancer, throat cancer, esophageal cancer, kidney cancer, colon cancer or any other type of cancer.

The invention also provides a method of determining the progression of a cancer comprising obtaining a biological sample containing a cell at a first time point from a patient diagnosed with cancer and obtaining a biological sample containing a cell from the patient at a second time point; contacting the cell in the first sample and the cell in the second sample with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell;determining the amount of cancer cells in both the first sample and the second sample; and comparing the amount of cancer cells in both the first sample and the second sample, whereby the relative amount of cancer cells in the first sample as compared with the second sample may be correlated with the progression of cancer, thereby determining the progression of the cancer.

The invention also provides a method of determining the progression of a cancer comprising obtaining a biological sample containing a cell at a first time point from a patient diagnosed with cancer and obtaining a biological sample containing a cell from the patient at a second time point; contacting the cell in the first sample and the cell in the second sample with a probe under conditions which allow the probe to form a complex with an antigen of the cell; detecting the complex in both the first sample and the second sample, whereby detection of the complex can distinguish a

non-cancer cell from a cancer cell; determining the amount of cancer cells in the first sample and the second sample; and comparing the amount of cancer cells in both the first sample and the second sample, whereby the relative amount of cancer cells in the first sample as compared with the second sample may be correlated with the progression of cancer, thereby determining the progression of the cancer.

The invention also provides a method of determining the progression of a cancer comprising obtaining a first biological sample from a patient diagnosed with cancer at a first time point and a second biological sample from a patient second time point, wherein the first and second biological sample comprise a mixed cell population suspected of containing a population of epithelial cells which include a cancer cell; mixing the first biological sample with magnetic particles and the second biological sample with magnetic particles, wherein the magnetic particles are coupled to a ligand which is capable of reacting specifically with epithelial cells to the substantial exclusion of non-epithelial cells; enriching the first biological sample and the second biological sample for epithelial cells by subjecting each mixture to a magnetic field to produce a first cell suspension that is enriched epithelial cells and a second cell suspension that is enriched epithelial cells; contacting each of the first sample of enriched cells and the second sample of enriched cells with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe for the first sample and the second sample, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the first sample and the second sample, and correlating the amount of cancer cells in the sample with the progression of cancer, thereby determining the progression of the cancer.

One skilled in the art is aware that by measuring the number of cancer cells in the patient at different intervals, the progression of the cancer can be determined. For example, if the patient is assayed for the presence of cancer cells at a first time point

and the number of cancer cells increases when the patient is assayed at a second time point, the skilled artisan would know the cancer has progressed. If the number of cancer cells decreases when the patient is assayed at a second time point, the skilled practitioner would know the cancer has not progressed. If the number of cancer cells remains the same when the patient is assayed at a second time point, the skilled practitioner would know the cancer has not progressed. Treatment regimens can, therefore, be adjusted correspondingly.

The invention also provides a method of determining the effectiveness of an anti-cancer treatment comprising obtaining a biological sample containing a cell from a patient that has been administered an anti-cancer treatment; contacting the cell in the sample with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with the effectiveness of the anti-cancer treatment, thereby determining the determining the effectiveness of an anti-cancer treatment.

The invention also provides a method of determining the effectiveness of an anti-cancer treatment comprising obtaining a biological sample containing a cell from a patient that has been administered an anti-cancer treatment; contacting the cell in the sample with a probe under conditions capable of forming a complex with an antigen of the cell; detecting the complex, whereby detecting the complex can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with the effectiveness of the anti-cancer treatment, thereby determining the determining the effectiveness of an anti-cancer treatment.

The invention further provides a method of determining the effectiveness of an anti-cancer treatment comprising obtaining a sample from a patient that has been administered an anti-cancer treatment, obtaining a biological sample from a subject, wherein the biological sample comprises a mixed cell population suspected of containing a population of epithelial cells which include a cancer cell; mixing the biological sample with magnetic particles coupled to a ligand which is capable of reacting specifically with epithelial cells to the substantial exclusion of non-epithelial cells; enriching the biological sample for epithelial cells by subjecting the mixture of the biological sample with magnetic particles coupled to a ligand to a magnetic field to produce a cell suspension that is enriched epithelial cells; contacting the enriched cells with a probe capable of hybridizing to nucleic acid of the cell; detecting the hybridization pattern of the probe, whereby the hybridization pattern can distinguish a non-cancer cell from a cancer cell; determining the amount of cancer cells in the sample and correlating the amount of cancer cells in the sample with the effectiveness of the anti-cancer treatment, thereby determining the determining the effectiveness of an anti-cancer treatment.

A person skilled in the art is aware that the methods of this invention can be utilized to test the efficacy of anticancer treatment. For example, if the patient diagnosed with cancer is assayed for the presence of cancer cells prior to the administration of an anticancer treatment and assayed at a second time point after the administration of the anticancer treatment, a decrease in the level of cancer cells may indicate an effective anticancer treatment had been administered. The skilled practitioner will associate the decreases observed with a particular level of effectiveness. If no decrease is observed, the anticancer treatment may be need to be adjusted.

For example, advanced primary breast cancer patients are treated preoperatively with adriamycin and Cytoxan and then monitored closely for a response. They usually

receive 2-3 cycles, 3 weeks apart before a determination of response or no response is made. If they respond, chemotherapy is continued. If they do not respond, a mastectomy is performed if it is technically feasible, or, if not feasible, the patient is switched to Taxol or Tacotere. By utilizing the methods of this invention, the changes in blood levels of breast carcinoma cells reflect changes in clinical status. By accurately determining the response to the treatment with a blood test using one of the methods described herein, tumor growth can be monitored and the response to chemotherapy can be determined more readily. In addition to breast cancer patients, the detection method can be used to identify and monitor other common carcinomas such as prostate, pancreatic, colon and lung cancers.

The sample of this invention can be from any organism and can be, but is not limited to, peripheral blood, bone marrow specimens, primary tumors, embedded tissue sections, frozen tissue sections, cell preparations, cytological preparations, exfoliate samples (e.g., sputum), fine needle aspirations, amnion cells, fresh tissue, dry tissue, and cultured cells or tissue. It is further contemplated that the biological sample of this invention can also be whole cells or cell organelles (e.g., nuclei). The sample can be unfixed or fixed according to standard protocols widely available in the art and can also be embedded in a suitable medium for preparation of the sample. For example, the sample can be embedded in paraffin or other suitable medium (e.g., epoxy or acrylamide) to facilitate preparation of the biological specimen for the detection methods of this invention. Furthermore, the sample can be embedded in any commercially available mounting medium, either aqueous or organic, depending on the chemical properties of the stain or any specifically developed medium, such as, for example, as designed for TMB, based on a thin protein layer cross-linked by formaldehyde to ensure permanent stabilization of the enzyme reaction products (*Speel et al.* 1994. "A novel triple-color detection procedure for brightfield microscopy, combining *in situ* hybridization with immunocytochemistry." J. Histochem. Cytochem. 42:1299-1307).

The sample can be on, supported by, or attached to, a substrate which facilitates detection of phenotypic or genetic markers. A substrate of the present invention can be, but is not limited to, a microscope slide, a culture dish, a culture flask, a culture plate, a culture chamber, DNA arrays, ELISA plates, as well as any other substrate that can be used for containing or supporting biological samples for analysis according to the methods of the present invention. The substrate can be of any material suitable for the purposes of this invention, such as, for example, glass, plastic, polystyrene, mica and the like. The substrates of the present invention can be obtained from commercial sources or prepared according to standard procedures well known in the art.

The sample to be assayed by the present methods can contain a cell that is derived from a patient, i.e., circulating cells, biopsy cells, scrapings, etc. The sample of the present invention can optionally be contacted with one or more cytological stains. The cytological stains used in the methods of this invention can be, but are not limited to, hematoxylin, eosin, methyl green, neutral red, DIFF QUIK (Baxter, The Netherlands), toluidine blue, alcian blue, isamin blue, methylene blue, sudan black, periodic acid-Schiff reaction (PAS), Masson's trichrome method, reticulin stain, Van Gieson, Azan, Giemsa, Nissl, silver and gold stains, osmium and chrom alum, as well as any other cytological stains now known or identified in the future. The cytological stains of this invention are available from commercial sources or can be prepared according to standard methods well known in the art.

It will become evident to one skilled in the art that the methods of this invention can be utilized to detect nucleic acids or antigens associated with the cancer cell. With respect to nucleic acids, the hybridization pattern identified by the methods of this invention can be, but are not limited to, those detected by hybridizing a nucleic acid probe to DNA, RNA or any other nucleic acid which can be, for example, tumor suppressor genes, oncogenes and proliferation markers. These hybridization patterns can be detected by methods well known in the art, including modifications of the

methods described herein to detect nucleic acid probes, such as binding to the nucleic acid probe a detectable molecule such as a nucleic acid, a hapten, a protein, an antigen, and an antibody, or fragments thereof. Examples of these techniques include Southern blot, Northern blot, dot blot, *in situ* hybridization or any other technique utilized to detect nucleic acids, including detecting nucleic acids that are immobilized on a solid support, e.g., cDNA arrays. Microscopy, fluorescence and radioisotopic detection methods can be also be used alone in combination with other nucleic acid detection methods to identify a hybridization pattern.

The complexes identified in the methods of this invention can be, but are not limited to gene products, antigens, antibodies, and proteins, or fragments thereof. Examples of gene products which can be detected include, but are not limited to, gene products of p53, retinoblastoma, Ki167, PCNA, nucleolus organizing regions and cyclins. Detection of the complex can be achieved by Western blot, immunohistochemistry, fluorescence, radiochemical or chromogenic methods.

The probes which are used to detect cancer cells by detecting an antigen associated with cancer, therefore, include any compound which can bind to, link to, hybridize to, or otherwise associate with the cancer cell. For example, the compound can be an antibody to a protein or a fragment of a protein, an antibody to a nucleic acid, an antibody to a ligand or a fragment of a ligand, an antibody to an antibody or fragment of an antibody (anti-idiotype antibody), an antibody to any cellular structure or fragment of the cellular structure, and the like. Alternatively, the compound can comprise other molecules such as nucleic acids, ligands, haptens, cell structures, and fragments thereof.

The present invention also provides detection methods wherein the probes are specific for genetic markers. The genetic markers of this invention can be, but are not limited to, centromeres, telomeres, general or specific loci, chromosome bands,

chromosome abnormalities, a chromosome-specific loci, chromosome fragments, and whole chromosomes, as well as any genetic marker which detects numerical chromosome alterations or structural chromosome alterations such as translocations, breakpoints, microdeletions and amplifications. For detection of these genetic markers, a nucleic acid probe having complementarity to the nucleotide sequence of the genetic marker is contacted with the biological sample under conditions whereby hybridization of the nucleic acid of the genetic marker and the nucleic acid probe can occur. These conditions can vary, depending of the biological sample, genetic marker and nucleic acid probe used for a given application. The hybridization conditions for a particular application can be determined according to protocols standard in the art.

One skilled in the art will recognize that there are many assays based on visualization of the genetic marker which can be used in the methods disclosed herein. For example, interphase *in situ* hybridization may be used to detect the presence of a chromosome abnormality which is associated with invasive cervical carcinoma or an advanced-stage cervical carcinoma. Interphase cytogenetics refers to the fact that chromosome aberrations can be analyzed directly in non-dividing cells, such as cytological preparations or in tissue sections. For instance, a trisomy 21 presents itself in metaphase chromosome of the specimen as three visible copies of this particular chromosome. A hybridization with a probe for chromosome 21 on the metaphase chromosome would highlight the three copies. The hybridization signal can be detected, for example, with fluorescent tags, or in a colorimetric format in which an absorbent color identifies the normal chromosome and the chromosome abnormality. In interphase cells, chromosomes have a morphology different than chromosomes in metaphase. The interphase chromosome, however, still maintains its organizational structure. Therefore hybridization of this interphase chromosome with a probe that recognizes chromosome 21 will highlight the additional chromosome by revealing an additional signal in the intact cells. Normal cells contain two copies; the super-numerary is indicated by three distinct spots. This approach, for example, can be used

for screening of Pap smears with a chromosome-specific probe, such as a 3q probe, to detect the presence of an invasive cervical carcinoma. The acquisition of additional copies of at least a fragment of a chromosome or chromosome abnormality identified as associated with the progression of cervical carcinomas can be visualized directly in intact cells after the hybridization with a probe specific for selected chromosome or chromosome fragment. One skilled in the art will therefore appreciate that the cancer cells detected by the claimed methods include interphase cells.

The detection of cancer cells and genetic markers in the sample can be combined with the routine assessment of histological and cytological specimens, generally carried out by staining with one or more cytological stains and examining the specimens microscopically. Thus, the present invention provides for multi-parameter analyses of the same biological sample.

A probe capable of hybridizing to nucleic acid of a cell can be a nucleic acid comprising the nucleotide sequence of a coding strand or its complementary strand or the nucleotide sequence of a sense strand or antisense strand. The nucleic acid can comprise the nucleic acid of a gene, or a sequence associated with a gene that is associated with cancer or the induction of cancer. Thus, the probe of this invention can be either DNA or RNA and can bind either DNA or RNA, or both, in the biological sample. The probe can be the coding or complementary strand of a complete gene or gene fragment. The nucleotide sequence of the probe can be any sequence having sufficient complementarity to a nucleic acid sequence in the biological sample to allow for hybridization of the probe to the target nucleic acid in the biological sample under a desired hybridization condition. Ideally, the probe will hybridize only to the nucleic acid target of interest in the sample and will not bind non-specifically to other non-complementary nucleic acids in the sample or other regions of the target nucleic acid in the sample. The hybridization conditions can be varied according to the degree of stringency desired in the hybridization. For example, if the hybridization conditions

are for high stringency, the probe will bind only to the nucleic acid sequences in the sample with which it has a very high degree of complementarity. Low stringency hybridization conditions will allow for hybridization of the probe to nucleic acid sequences in the sample which have some complementarity but which are not as highly complementary to the probe sequence as would be required for hybridization to occur at high stringency. Since sequence divergence can exist between individuals for cancer or tumor-related genes, one skilled in the art can take these population differences into account when optimizing hybridization conditions. The hybridization conditions will therefore vary depending on the biological sample, probe type and target. An artisan will know how to optimize hybridization conditions for a particular application of the present method.

The nucleic acid probes of this invention can be modified nucleic acids. These modified nucleotides are well known in the art and include, but are not limited to, thio-modified deoxynucleotide triphosphates and borano-modified deoxynucleotide triphosphates (Eckstein and Gish, *Trends in Biochem. Sci.*, 14:97-100 (1989) and Porter *Nucleic Acids Research*, 25:1611-1617 (1997)).

The nucleic acid probe can be commercially obtained or can be synthesized according to standard nucleotide synthesizing protocols well known in the art. Alternatively, the probe can be produced by isolation and purification of a nucleic acid sequence from biological materials according to methods standard in the art of molecular biology (Sambrook et al. 1989. *Molecular Cloning: A Laboratory Manual*, 2d Ed., Cold Spring Harbor Laboratory Pres, Cold Spring Harbor, NY). The nucleic acid probe can be amplified according to well known procedure for amplification of nucleic acid (e.g., polymerase chain reaction). Furthermore, the probe of this invention can be linked to any of the detectable moieties of this invention by protocols standard in the art.

The detectable moieties to which the nucleic acid probe of this invention can be linked to include, but are not limited to, a hapten, biotin, digoxigenin, fluorescein isothiocyanate (FITC), dinitrophenyl, amino methyl coumarin acetic acid, acetylaminofluorene and mercury-sulfhydryl-ligand complexes, as well as any other molecule or compound which can be linked to a probe and detected either directly or indirectly according to the methods described herein.

One skilled in the art will therefore appreciate that a probe, such as a nucleic acid probe or an antibody probe, can be labeled with a detectable moiety that can be directly detected, such as a flurorochrome or a dye, such as a chromogenic dye, and the use of secondary reagents to detect the probe is not strictly required.

It is further contemplated that the present invention also includes methods for oligonucleotide hybridization wherein the hybridized oligonucleotide is used as a primer for an enzyme catalyzed elongation reaction such as *in situ* PCR and primed *in situ* labeling reactions whereby haptenized nucleotides are incorporated *in situ*. Additionally included are methods for *in situ* hybridization, employing synthetic peptide nucleic acid (PNA) oligonucleotide probes (*Nielsen et al.*, 1991. "Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide." Science 254:1497-1500; *Egholm et al.*, 1993. "PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen bonding rules." Nature 365:566-568).

The methods described herein can use, for example, spectral imaging for the specific detection method. Multicolor spectral karotyping (SKY) of human chromosomes is a spectral imaging approach that combines Fourier spectroscopy, charge-coupled device imaging, and optical microscopy to measure simultaneously all points in the emission spectra in the visible and near-infra-red spectral range (*Schrock et al.* 1996 "Multicolor spectral karotyping of human chromosomes" *Science*, 273:494-

497). This allows the use of multiple spectrally overlapping probes (*Schrock et al.*; *Ried et al.* 1995 "Comparative genomic hybridization of formalin-fixed, paraffin-embedded breast tumors reveals different patterns of chromosomal gains and losses in fibroadenomas and diploid and aneuploid carcinomas." Cancer Res. 55:5415-5423; *DeRisi et al.* 1996. "Use of a cDNA microarray to analyze gene expression patterns in human cancer" Nature Genetics 14: 457-460; *Coleman et al.* 1997. "Previously hidden chromosome aberrations in T(12;15)-positive BALB/c plasymacytomas uncovered by multicolor spectral karotyping." Cancer Res. 57:4584-4592; *Garini et al.* In Flourescence Imaging Spectroscopy and Microscopy, X.F. Wang and B. Herman, Eds. (Wiley, New York, 1996), 137:87-124; and *Ried* 1998. "Interphase cytogenetics and its role in molecular diagnostics of solid tumors." American J. of Pathology 152:325-327.

Emission spectra are then converted to colors by assigning blue, green, and red colors to specific spectral ranges. Chromosome-specific labeling is achieved by suppression hybridization. Specifically, repetitive sequences in the composite libraries are blocked by the addition of an excess of unlabeled human DNA enriched for repetitive sequences (Cot-1 DNA). The result is that every human chromosome can be painted a different color. Most important is that the technique can be applied to interphase cells (*Ried*, 1998). As many as 10 chromosomes can be probed in a single cell preparation. This provides sensitive detection and precise enumeration of the cell pool that carries specific chromosomal abnormalities. The technique has detected many genetic changes in breast cancer but certain changes predominate (*Ried et al*, *De Risi et al.*) Multi-color FISH (MCF) using >1 probe can be used on immunohistochemically-stained interphase cells on slides, MCF is, therefore, an effective screening test for copy number changes in tumor genomes and for detection of genomic imbalances in breast carcinoma cells in blood.

In the present invention, the spectral image of the biological sample on the substrate can be obtained with a device which utilizes a common path Sagnac

interferometer creating an optical path difference based on the angle of incident light. An interferogram is produced showing the light intensities against the function of the optical path difference. Fourier transformation of the interferogram recovers the spectrum. An example of this device is a SD200 Spectracube (Applied Spectral Image, Migdal HaEmek, Israel). Other methods to measure absorption spectra using light microscopy can include spectrophotometry, the use of liquid crystal tunable filters and accusto-optical tunable filters. The spectral image of the biological sample of this invention can be analyzed with software designed for spectral image analysis, such as the SpCube 1.5 program (Applied Spectral Imaging).

Detection of cancer cells and genetic markers in a sample can be by microscopy, which can be, but is not limited to, bright-field microscopy, phase contrast microscopy, interference contrast microscopy, Nomarski contrast microscopy, dark field microscopy, reflection contrast microscopy, fluorescence microscopy, infra-red microscopy, or any other type of light microscopy. Detection can be done by visualizing the biological sample in the microscope or by recording an image of the biological sample photographically (e.g., by producing an image on a silver halide emulsion film which can be developed for visualization or by recording a digital image of the sample for visualization via an output device, such as, for example, a computer monitor or as a computer printout) as well as by any other means by which the biological sample can be viewed or recorded. The spectral image of the biological sample can be taken from the sample directly or from a recorded image of the sample.

In another method of detection, the probe can be directly detected by linking a detectable moiety to the probe or compound which can facilitate direct detection, such as an enzyme (e.g., peroxidase, alkaline phosphatase, glucose oxidase) which produces a colored reaction product when reacted with a suitable substrate or to colloidal gold particles or other detectable moieties. Alternatively, the probe can be detected indirectly by the binding of antibodies, antibody fragments or other ligands, or the

reaction of other molecules (*e.g.*, avidin to detect biotin) with the probe, including for example, enzymes such as peroxidase, alkaline phosphatase or glucose oxidase for enzymatic precipitation upon reaction with suitable substrates to produce a colored reaction product, *i.e.*, a chromogenic dye associated with the detectable moiety. Similarly, the detectable moeity may be associated with a fluorescent dye. As used herein, "associated with a chromogenic (or fluorescent)dye" includes being covalently, ionically, or electrostatically bound to the chromogenic or fluorescent dye. The enzyme peroxidase can also be used in conjunction with tyramide-based detection formats. Other detection methods include fluorescence detection methods and radioisotopic detection methods, which are well known in the art.

For example, the antibodies, antibody fragments or ligands can also be linked to colloidal gold particles for direct detection or subsequently enhanced with silver for indirect detection. The detectable moieties of this invention are available from commercial sources or can be prepared according to standard protocols well known in the art. Methods for detecting the detectable moieties of the present invention are common in the art. Protocols for linking probes, detectable moieties, antibodies, ligands, etc., are also standard in the art and are readily available to the artisan. Additionally, the detectable moieties exemplified here can be detected in any number of alternative detection procedures other than those listed.

The detectable moiety of this invention can also comprise an antibody. The antibody can be either monoclonal or polyclonal. The antibodies of this invention can also include immunoreactive antibody fragments. The detectable moiety can also comprise a ligand or any other molecule that can detect the antibody or the nucleic acid probe.

Antibodies can be made by many well-known methods (See, e.g. Harlow and Lane, "*Antibodies; A Laboratory Manual*" Cold Spring Harbor Laboratory, Cold

Spring Harbor, New York, (1988)). Briefly, purified antigen can be injected into an animal, with or without adjuvants, in an amount and in intervals sufficient to elicit an immune response. Polyclonal antibodies can be purified directly, or spleen cells can be obtained from the animal for monoclonal antibody production. The spleen cells can be fused with an immortal cell line and the resulting hybridomas can be screened for antibody secretion. A variety of immunoassay formats can be used to select antibodies which selectively bind with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies selectively immunoreactive with a protein. See Harlow and Lane (1988) for a description of immunoassay formats and conditions that can be used to characterize antibody binding.

In some instances, it is desirable to prepare monoclonal antibodies from various hosts, for example, for anti-species antibodies. A description of techniques for preparing such monoclonal antibodies may be found in *Stites et al.*, editors, "Basic and Clinical Immunology," (Lange Medical Publications, Los Altos, Calif., Fourth Edition) and references cited therein, as well as in Harlow and Lane (1988).

As described above, the antibody of the present invention can bind an antigen which is attached to the nucleic acid probe. The antibody itself can be linked to a detectable moiety, such as an enzyme, and binding of antibody to an antigen attached to a nucleic acid probe can thereby be detected directly. Alternatively, the antibody which binds the antigen which is attached to the nucleic acid probe can be detected indirectly, by binding a second antibody which recognizes the first bound antibody as an antigen. The second antibody can be linked to a detectable moiety, such as an enzyme, thereby detecting the binding of the first antibody indirectly.

The invention also contemplates the use of multiple probes in detection methods. For example, one probe can be directed toward a genetic marker that identifies the cell as cancer cell and another probe can be directed toward a genetic

marker that would identify the organ origin of the tumor cell. For example, in order to identify breast cancer cells, the cells could be probed with anti-cytokeratin as well as anti-Mucin 1. Since breast cancer cells, but not normal epithelial cells stain for Mucin 1, those cells stained with both cytokeratin and Mucin 1 would be of breast tumor origin. In utilizing multiple probes, more than one probe can be to different genetic markers, the same genetic marker, or more than one genetic marker and more than one phenotypic marker.

The invention further provides methods for detecting cancer cells, tumor cells, circulating cells, epithelial cells, circulating epithelial cells, cervical cancer cells, breast cancer cells, prostate cancer cells, lung cancer cells, pancreatic cancer cells, brain cancer cells, ovarian cancer cells, liver cancer cell, throat cancer cells, esophageal cancer cells, kidney cancer cells, colon cancer cells or any other cell associated with any cancer or tumorigenesis.

In one example, the cells in the sample can be assayed using probes detected using comparative genomic hybridization (CGH). This type of assay serves as a screening test for DNA-copy number changes in tumor genomes. CGH is based on a two color fluorescence in situ hybridization, where a normal reference genome is labeled with a first fluorochrome (e.g. rhodamine) and genomic tumor DNA with a second fluorochrome, (e.g. fluorescein). After the hybridization of the differentially labeled genomes to normal reference metaphase chromosomes the changes in the ratio of the fluorescein/rhodamine intensities reflect DNA-copy number alterations in the tumor. (See, e.g., Heselmeyer et al. "Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix" Proc. Nat. Acad. Sci. 93:479-484 (1996))

There are numerous chromosome and chromosome specific probes that can be used for detecting several human cancers. These include probes for cervical cancer that

detect a gain of chromosome 1q, 3q, 5p or 20 as well as probes that detect the loss of chromosome 3p or 13q. (*Heselmeyer et al.* 1996 "Gain of chromosome 3q defines the transition from severe dysplasia to invasive carcinoma of the uterine cervix" Proc. Natl. Acad. 93:497-484 and *Heselmayer et al.* 1997 "Advanced stage cervical carcinomas are defined by a recurrent pattern of chromosomal aberrations revealing high genetic instability and a consistent gain of chromosome 3q" Genes Chromosom. Cancer 19:233-240.) Probes for pancreatic cancer that detect the gain of chromosome 5p,7,8q or 20q as well as probes that detect the loss of chromosome 8p, 9p or 18q in pancreatic cancer are also available (*Ghadimi et al.* 1999 "A recurring pattern of chromosomal gains and losses and amplification of the AIB-1 steroid receptor gene in pancreatic carcinomas identified by comparative genomic hybridization and spectral karotyping" Am. J. Pathol. 154:525-536). Probes that detect the gain of chromosome 7p, 8q, 13 or 20 as well as probes that detect the loss of chromosome 8p or 18q are available for the detection of colorectal carcinomas (*Ried et al.* 1996 "Comparative genomic hybridization reveals a specific pattern of chromosomal gains and losses during the genesis of colorectal tumors" Genes Chromosom. Cancer 15:234-245). Probes for the detection of breast cancer include probes that detect the gain of chromosome 81, 5p, 17q, or 20q as well as probes that detect the loss of chromosomes 8p, 16p or 17p (*Ried et al.* 1995 "Comparative genomic hybridization of formalin fixed, paraffin embedded breast carcinomas reveals different patterns of chromosomal gains and losses infibroadenomas and diploid and aneuploid carcinomas" Cancer Res. 55:5415-5423 and *Kallioniemi et al.* 1994 "Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization" Proc. Natl. Acad. Sci. USA 91:2156-2160). Probes available for detection of lung cancer include probes that can detect the gain of chromosome 3q, 5p, 8q, or 19q as well as probes that can detect the loss of chromosomes 3p, 5q, 13 and 17p (*Petersen et al.* 1997 "Small cell lung cancer is characterized by a high incidence of deletions on chromosomes 3p, 4q, 5q, 10q, 13q and 17p" Brit. J. Cancer 75:79-86, *Schwendel et al.* 1997 "Primary small cell lung carcinomas and their metastases are characterized by a recurrent pattern of genetic

alterations" Int. J. Cancer 20:86-93, and *Petersen et al.* 1997 "Patterns of chromosomal imbalances in adenocarcinomas and squamous cell carcinomas of the lung" Cancer Res. 57:2331-2335). Prostate specific probes include probes that can detect a gain of chromosome 7, 8q or X as well as probes that can detect a loss in chromosome 8p, 13q, 6q or 16q (*Visakorpi et al.* 1995 "Genetic changes in primary and recurrent prostate cancer by comparative genomic hybridization" Cancer Res. 55:342-347). Probes for the detection of ovarian cancer include probes that can detect a gain in chromosome 3q, 8q or 20q as well as probes that can detect a loss in chromosome 16q, 17p or 17q (*Iwabuchi et al.* 1995 "Genetic analysis of benign, low-grade, and high-grade ovarian tumors" Cancer Res. 1995:6172-80). One skilled in the art will, therefore, recognize that many examples of chromosome alterations are associated with a specific cancer which can therefore be used to detect a cancer cell and/or determine the organ-origin of the cancer cell.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the claims included herein.

EXAMPLES

In one example of the present methods, circulating epithelial cells from blood specimens of control and cancer bearing patients can be enriched by methods such as magnetic separation using specific cell-specific antigen/antibody complexes and characterized by staining with specific antibodies and using methods such as flow cytometry and immunohistochemistry. For example, *Racila et al.* (*Proc. Nat. Acad.*

Sci. USA 95:4589-4594 (1998)) demonstrate using cytokeratin monoclonal antibodies to enrich for circulating epithelial cells followed by immunological detection of cell surface markers. Specifically, the intact cells can be exposed to a combination of monoclonal antibodies which can be in turn linked to detectable moieties to identify individual cells as epithelial cells.

For example, Figure 1 shows cells that were stained with an antibody against keratin (arrows). The large red-stained cells indicate the presence of epithelial cells in peripheral blood and the smaller, unstained cells (arrowheads) represent lymphocytes. This procedure has the capacity to detect whether epithelial cells are present in a sample, such as blood, but does not indicate the genetic status of the cells detected. Therefore further assays on the same sample are required to determine more conclusively the genetic status of these epithelial cells.

Using the methods provided herein, the cells in the sample that would otherwise only indicate whether cancer cells are present can further determine the genetic status of those cells. Cells not containing the normal genetic content as normal cells, i.e., aneuploid cells, indicates that those cells are cancer cells, whether or not those cancer cells are removed from their original location, such as in or near a tumor. Figure 2 shows, for example, cytokeratin-negative control cells and cytokeratin-positive cells that were assayed using in situ hybridization of a fluorescently labeled DNA probe for the centromere of chromosome 8. The control cell exhibits the normal number of signals for the centromere of chromosome 8, whereas the cytokeratin-positive cell is definitively characterized as an aneuploid cell, and therefore a cancer cell, since the number of hybridization signals is twice that of the control cell. This method, therefore, definitively identifies the cell as a cancer cell, rather than just a circulating epithelial cell.

To exemplify the methods claimed herein in further detail, the following experimental procedure is provided in which fluorescent in situ hybridization was performed on cells to demonstrate they are circulating epithelial cells that contain aberrant genomes and are cancer cells:

Circulating cells were obtained from patients clinically diagnosed as having prostate cancer or breast cancer. The cells obtained for this example were first obtained from patient blood samples via conventional clinical procedures. The cells were mixed with magnetic beads coated with anti-cytokeratin antibodies and isolated from the cells in the sample not expressing cytokeratin via methods well known in the art, such as centrifuging the magnetic beads and decanting the supernatant containing the cells not expressing cytokeratin. Methods such as this for isolating cytokeratin-expressing cells are known to those skilled in the art and are not limited to this example. For example, other tumor-specific antigens can be used in similar methods to isolate tumor cells from a blood sample, or other samples such as saliva, tissue, mucus, or samples where the cells have been previously immobilized and/or fixed. One skilled in the art will appreciate that the present methods are not limited by the source of the samples containing the cells that are assayed.

The nucleic acid probe can be prepared in a number of ways that one skilled in the art will recognize, but a specific example is as follows:

Nick-Translation of the probe:

For each DNA sample, 2 μg DNA is mixed with 10 μl 10xNT-Buffer, 10 μl dNTP (0.5 mM each of dATP, dCTP, dGTP and 0.05 mM dTTP), 10 μl 0.1 M ß-Mercaptoethanol, 4 μl BIO-11-dUTP or 4 µl DIG-11-dUTP or 6 µl of FITC-12-dUTP or 6 µl of TR-5-dUTP (1 mM), and sterile water to a total final volume of 100μl (see directly below). The mixture is vortexed, centrifuged, and kept on ice. 0.5-3 μl DNAse

(1mg/ml) 1:1000 and 2 μl Polymerase (Komberg). The samples are again vortexed and centrifuged and incubated at 15°C for 1.5-2 hr.

Approximately 5 µl of each sample is electrophoresed to determine the fragment size; ideally the length of the DNA should be between 300-600bp after Nick translation. If the DNA is too large, add more DNase and incubate at 15°C for 10-20 minutes, as necessary. When the DNA is of the appropriate size, terminate the Nick translation with 1 μl of 0.5 M EDTA and incubate at 65°C for 10 minutes. Store DNA at -20°C or precipitate the same day.

Slide Pretreatment:

Solutions:

RNase A: Boehringer, 109169, 100 mg
stock solution: 20 mg/ml sterile water, boil for 15 min, cool to room temp., make aliquots, store at -20 °C

Pepsin: Sigma, P 6887, 5g
stock solution: 10 % = 100 mg/ml, dissolve in sterile water, keep on ice, make 50 µl aliquots, store at - 20 °C

PBS/$MgCl_2$: 50 ml 1M $MgCl_2$ + 950 ml 1xPBS

I. Equilibrate in 2X SSC at room temperature the slides having attached cells from a sample

II. RNase - treatment:

1. Dilute the RNase stock 1:200 (in 2 x SSC)

2. Apply 100-200 µl to 24x60 mm^2 coverslip, touch slide to coverslip, incubate at 37°C for 45 - 60 mins

3. Remove coverslips and wash 3 x 5 minutes in 2 x SSC, RT shaking (Coplin jar)

III.Pepsin -treatment:

1. Prepare solution: make 0.01 HCl by adding about 1ml 1M HCl to 99ml dH_20, prewarm at 37°C, add 10 - 50µl Pepsin first then add 100ml prewarmed 0.01 M HCl, mix well and adjust pH to 2.0

2. Incubate slides at 37°C in coplin jar for 4-10 mins

3.Wash 2 x 5 mins in 1x PBS, RT, shaking

4.Wash 1 x 5 mins in 1x $PBS/MgCl_2$

IV. Postfixation

1. Make solution of 1% Formaldehyde in 1 x $PBS/MgCl_2$ (add 2.7ml of 37% Formaldehyde to 100ml 1 x $PBS/MgCl^2$)

2. Incubate for 10 minutes, RT

3. Wash 1 x 5 min in 1 x PBS, RT, shaking

4. Dehydrate slides in 70, 90, 100% Ethanol for 3 minutes each

5. Air dry slides

The pepsin incubation times and concentration vary depending on the age of slides and contamination with remaining cytoplasm and cell debris around the chromosomes. Too harsh treatment can result in chromosome damage and subsequently bad hybridization quality whereas too mild treatment can result in low hybridization to noise signals.

DNA precipitation and hybridization for FISH

Add to an eppendorf tube: 15-30 µl nick-translated probe DNA (300-600 ng DNA), 20µl Human Cot-1™ DNA (1mg/ml),1µl salmon sperm DNA (10 mg/ml), {Human Cot-1™ DNA 500µg (1mg/ml): GIBCO BRL: cat. # 15279-011}, {Salmon Testes DNA 1ml 9.7mg/ml: SIGMA Molecular Biology: cat. # D-7657}. Add 1/10 volume Na-Acetate (3M) and 2.5 -3.0 x total volume of 100% Ethanol. Vortex and store at -20 °C overnight or at -80° C for at least 15- 30 min. Centrifuge (13,000 rpm) precipitated DNA at 4 °C for 30 min. Pour off supernatant and speed vac for 5 min to dry pellet. Add 5µl deionized Formamide (pH 7.5), incubate at 37 °C for 30 min, vortexing periodically. Add 5 µl of prewarmed Master Mix, mix carefully before adding, vortex, spin briefly (MM - 20% dextran sulfate in 2 x SSC, pH 7.0, autoclaved, stored at - 20 °C). Denature the probe DNA at 76 °C for 5 min, spin briefly. Preanneal at 37 °C for 1-2 hrs. For slide denaturation apply 100 µl 70% Formamide/2 x SSC (70 µl deionized FA, 3 µl 20 x SSC, 27 µl Sterile Water, pH 7.5) to a 24x60mm^2 coverslip, touch slide to coverslip. Incubate slides at 75°C for 1.5 min. Immediately place in ice cold 70% Ethanol for 3 min, followed by 90% Ethanol and 100% Ethanol for 3 min each, air dry. Add probe DNA after preannealing to denaturated slides and cover with 18 mm^2 coverslips, seal coverslips with rubber cement.

FISH-Detection

Solutions: (use approximately 70 ml for glass coplin jars and 50 ml for plastic jars per step)

FA/SSC:

30 ml 20 x SSC
120 ml Sterile Water
150 ml Formamide
adjust pH to 7-7.5 by adding 1M HCl
(200ml of FA/SSC requires 500µl of 1 M HCl)

heat for 30 minutes at 45°C

4 x SSC/Tween 20:

100ml 20 x SSC

400ml H_20

0.5ml Tween 20

heat for 30 minutes at 45°C

0.1 x SSC:

2.5 ml 20 x SSC

add H_20 so final volume is 500 ml

heat for 30 minutes at 60°C

Blocking Solution(3% BSA):

add 0.3g Bovine Serum Albumin(powder) to pre-warmed (37°C)

10ml 4XSSC/Tween 20

vortex until dissolve at 37°C

Remove rubber cement and coverslips from hybridized slides. Wash slides in FA/SSC (use coplin jars), 3 x 5 min, shaking : First dip the slides into FA/SCC to remove coverslips. Next, wash slides in 0.1 x SSC, 3 x 5 min, while shaking. Dip the slides in 4 x SSC/Tween 20, being careful not to let it dry. Add 100 µl of Blocking Solution to 24 x 60 mm^2 coverslips, touch slides to coverslips and incubate in hybridization chamber in 37°C water bath for about 30 minutes. Dip the slides in 4 x SSC/Tween 20, being careful not to let it dry. (Note: Spin all fluorescent dyes for 3 min at 13,000 rpm)

Antibodies: Add 120 µl of prepared antibody solution (Antibodies should be diluted in 1% BSA/4xSSC/Tween) per coverslip (use 24 X 60 mm^2), touch slide to

coverslip and incubate in hybridization chamber for 45 minutes at 37°C. Wash slides in 4 x SSC/Tween 20, 3 x 5 minutes, while shaking. Apply a second directed toward the first antibody. DAPI staining for 5 min: stain slides in foil- covered coplin jar, use 8 ng/ml DAPI (4 μl of the stock solution to 100ml of 2XSSC) in 2x SCC (stock solution: 2 mg DAPI/10 ml Sterile Water), wash in H_20 for 5 min, while shaking, dehydrate in 70%, 90% and 100% ethanol for 3x3 min. Air dry and apply 30-35 µl antifade, cover with 24 X 60 mm^2 coverslips, store in dark at 4°C.

Antibodies for FISH (if not direct dyes used):

Avidin-FITC (1:200) (when Biotin used), mouse-anti-DIG (Sigma) (1:100) and rabbit anti mouse TRITC (1:200).

Figures 3 and 4 demonstrate the effectiveness of the described method. The cells in figure 3 were obtained from a patient with a prostate cancer. The cells were hybridized to centromere probes for chromosome 7 (red) and for chromosome 8 (green). The larger cell is a tumor cell which exhibits greater than two fluorescent *in situ* hybridization (FISH) signals whereas the normal cells show only two FISH signals.

Figure 4 shows a sample containing two cells from a woman with breast cancer. The cells were hybridized to centromere probes for chromosome 7 (red) and for chromosome 8 (green). The larger cell is a tumor cell which exhibits greater than two fluorescent *in situ* hybridization (FISH) signals whereas the normal cells show only two FISH signals.

These data clearly indicate that the present methods can detect circulating cancerous epithelial cells in a mixed population of cells. These results have been verified in three patients; two breast cancer patients and one patient with prostate cancer.

In order to validate the approach we have hybridized blood samples from 25 patients with breast cancer, and of 10 patients with benign disease and normal controls. In all but one of the breast cancer patients we detected cells with abnormal chromosome counts using interphase FISH with centromere probes or with probes for the c-myc and her2neu oncogene. Chromosomal aberrations were not detected in the controls. Most importantly, some of the breast cancers were at a very early stage with no lymph node involvement or distant metastases.

The results were all verified when we hybridized the same probes used for the cells in the peripheral blood on tumor sections after the patients had undergone surgery. The pattern detected could be matched in all but one case, This means that the cells in the peripheral blood were indeed derived from the tumor. It has also unexpectedly been discovered that it is possible to perform immunocytochemistry on a sample, i.e., treat the sample with a labeled antibody against cytokeratin, and to perform FISH on these same samples. Typically, samples that have been immunocytochemically stained for bright field microscopy would not be expected to be useful for subsequent FISH analysis (which of course uses fluorescence microscopy) due to interference between the different types of dyes used for these two microscopy techniques. However, using the methods of the invention, we were able to simultaneously visualize the epithelial origin of the cells and the chromosome count. This provides an internal control for the hybridization and increases the diagnostics accuracy.

Throughout this application various publications are referenced. The disclosures of all of these publications and those references cited within those publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.